Coexisting cryptic species of the Litoditis marina complex (Nematoda) show differential resource use and have distinct microbiomes with high intraspecific variability

Derycke, S.; De Meester, N.; Rigaux, A.; Creer, Simon; Bik, Holly M.; Thomas, W.K.; Moens, T.

Molecular Ecology

DOI:
10.1111/mec.13597

Published: 01/05/2016

Peer reviewed version

Title page

1. **Title:** Coexisting cryptic species of the *Litoditis marina* complex (Nematoda) show differential resource use and have distinct microbiomes with high intraspecific variability
2. **Authors:** Derycke S, De Meester N, Rigaux A, Creer S, Bik H, Thomas WK, Moens T
3. **Postal addresses:**

Derycke Sofie; OD Taxonomy and Phylogeny, Royal Belgian Institute of Natural Sciences, Vautierstraat 29, 1000 Brussels, Belgium; Department of Biology, Marine Biology Section, Ghent University, Krijgslaan 281 (S8), 9000 Ghent, Belgium; s.derycke@ugent.be

De Meester Nele; Department of Biology, Marine Biology Section, Ghent University, Krijgslaan 281 (S8), 9000 Ghent, Belgium; CeMoFE, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium; Nele.demeester@ugent.be

Rigaux Annelien; Department of Biology, Marine Biology Section, Ghent University, Krijgslaan 281 (S8), 9000 Ghent, Belgium; CeMoFE, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium; Annelien.rigaux@ugent.be

Creer Simon; Environment Centre Wales Building, School of Biological Sciences, Bangor University, Gwynedd, LL57 2UW; s.creer@bangor.ac.uk

Bik Holly; Center for Genomics and Systems Biology, New York University, 12 Waverly Place, New York, NY 10003, US; hbik@nyu.edu

Thomas William Kelley, Hubbard Center for Genome Studies, University of New Hampshire, 35 Colovos Road, 448 Greg Hall, Durham, New Hampshire 03824, US; Kelley.Thomas@unh.edu

Moens Tom; Department of Biology, Marine Biology Section, Ghent University, Krijgslaan 281 (S8), 9000 Ghent, Belgium; Tom.moens@ugent.be

4. **Keywords:** individual niche specialization, resource partitioning, coexistence, marine nematodes, bacteria, diet, next generation sequencing, stabilizing effects

5. **Corresponding author**

Dr. Sofie Derycke,

OD Taxonomy and Phylogeny, Royal Belgian Institute of Natural Sciences

Vautierstraat 29, Brussels, Belgium

Telephone: 0032 2 264 44 28

Email: sofie.derycke@naturalsciences.be

6. **Running title:** Microbiome differentiation among cryptic species

II. Abstract page

Differences in resource use or in tolerances to abiotic conditions are often invoked as potential mechanisms underlying the sympatric distribution of cryptic species. Additionally, the microbiome can provide physiological adaptations of the host to environmental conditions. We determined the intra- and interspecific variability of the microbiomes of three cryptic nematode species of the *Litoditis marina* species complex that co-occur, but show differences in abiotic tolerances. Roche 454 pyrosequencing of the microbial 16S rRNA gene revealed distinct bacterial communities characterized by a substantial diversity (85 – 513 OTUs) and many rare OTUs. The core microbiome of each species contained only very few OTUs (2 – 6), and four OTUs were identified as potentially generating tolerance to abiotic conditions. A controlled experiment in which nematodes from two cryptic species (Pm1 and Pm3) were fed with either an *E. coli* suspension or a bacterial mix was performed and the 16S rRNA gene was sequenced using the MiSeq technology. OTU richness was 10 fold higher compared to the 454 dataset and ranged between 1118 – 7864. This experiment confirmed the existence of species-specific microbiomes, a core microbiome with few OTUs, and high interindividual variability. The offered food source affected the bacterial community and illustrated different feeding behavior between the cryptic species, with Pm3 exhibiting a higher degree of selective feeding than Pm1. Morphologically similar species belonging to the same feeding guild (bacterivores) can thus have substantial differences in their associated microbiomes and feeding strategy, which in turn may have important ramifications for biodiversity – ecosystem functioning relationships.

58 **III. Main text**

59 (a) Introduction

60 Many taxa contain species that are morphologically (nearly) identical but show genetic
61 differences in neutral markers that are comparable to, or greater than, those observed between
62 species with distinct morphologies. These cryptic species have been observed in all major
63 taxa and in all biogeographic regions (Pfenninger & Schwenk 2007). Despite their
64 morphological similarity, cryptic species can have distinct evolutionary histories of millions
65 of years (Elmer *et al.* 2013; Glasby *et al.* 2013; Perez-Portela *et al.* 2013). The conservation
66 of the morphological pattern results from selection-promoting morphological stasis and/or
67 from a differentiation in other characters that are invisible to the human eye (Bickford *et al.*
68 2007). In the marine environment, cryptic species of benthic invertebrates often show a
69 sympatric distribution, but at the same time pronounced habitat preferences defined by depth,
70 salinity, temperature and substrate (Knowlton 1993). Next to these abiotic parameters,
71 intrinsic differences between cryptic species, such as the differential use of resources or the
72 presence of distinct microbiomes, may impact the sympatric distribution of cryptic species as
73 microbiomes can affect the physiology of the host (Cabreiro & Gems 2013; Sison-Mangus *et*
74 *al.* 2014) which may have cascading effects on ecological interactions.

75 Substantial cryptic diversity has been observed in the phylum Nematoda (de Leon & Nadler
76 2010; Derycke *et al.* 2013; Ristau *et al.* 2013; Sudhaus & Kiontke 2007). In marine
77 sediments, nematodes abound both in numbers and in local species diversity, with several tens
78 of species co-occurring at submeter scales (Heip *et al.* 1985). Nematode community
79 composition, assessed through morphological characters, can be linked to physico-chemical
80 characteristics of the sediment (Steyaert *et al.* 1999; Vanaverbeke *et al.* 2000), and at very

small spatial scales, microhabitat differences can substantially alter nematode communities (Fonseca *et al.* 2010; Gingold *et al.* 2011). Based on the shape of the buccal cavity and the presence/absence of armature in the stoma, marine nematodes have been divided into feeding guilds (Moens & Vincx 1997; Wieser 1953). Nematodes without buccal armature can feed on bacteria and protists, while those having buccal armature can feed on microalgae (e.g. diatoms), on micro-invertebrates including nematodes and on other resources (Moens & Vincx 1997). The niches of nematode species delineated by morphology are thus determined by a series of abiotic and biotic parameters, but the extent of niche breadth of, and niche differences between sympatrically occurring cryptic nematode species remain unknown. Moreover, the nematode microbiome influences the physiology of the worm and impacts its longevity (Cabreiro & Gems 2013) and may, especially in the case of bacterivorous nematodes, be linked to the diet of the nematodes. Techniques currently available to assess resource use in minute organisms (e.g. stable isotope analysis) are unable to distinguish individual resource use (Carman & Fry 2002). The advances in high throughput sequencing now allow to more deeply investigate the microbial communities associated with sympatric bacterivorous nematode species to determine the extent of resource differentiation (bacteria related to food) and of microbiome differentiation (the microbiome 'sensu lato', which comprises the bacteria related to food and the microbiome 'sensu stricto' containing the commensal bacteria).

The bacterivorous marine nematode *Litoditis marina* (Bastian, 1865) Sudhaus, 2011 consists of at least 10 cryptic species (Derycke *et al.* 2008b), three of which (Pm1, Pm2 and Pm3) frequently co-occur on seaweed stands and deposits in the coastal area of Belgium and The Netherlands (Derycke *et al.* 2005). In this region, the most abundant seaweeds typically belong to the genus *Fucus.* Phylogenetic analyses of mitochondrial and nuclear genes have

105 revealed that Pm3 is more distantly related to Pm1 and Pm2 (Derycke *et al.* 2008b).
106 Morphological differentiation between the three species is limited and requires a combination
107 of morphometric characters (Derycke *et al.* 2008a). No cross breeding between the species
108 has been observed under laboratory conditions (Fonseca *et al.* 2008; Derycke, unpublished
109 data). Their coexistence implies that local populations of the three sympatric species
110 experience (nearly) identical sets of abiotic factors like salinity and temperature. Nevertheless,
111 both factors differentially impact demographic traits of the three species, resulting in a
112 significantly lower generation time at higher temperatures and the production of more
113 offspring at lower salinities for Pm3 (De Meester *et al.* 2015b). Whether these species have a
114 microbiome and whether such a microbiome would differ between species remains unknown.
115 Furthermore, competitive interactions have been observed between these cryptic species (De
116 Meester *et al.* 2011) and the presence of a bacterial food source impacted their dispersal
117 behavior (De Meester *et al.* 2012). In addition to abiotic factors, niche differentiation between
118 the cryptic species may thus be linked to resource divergence. Chemotaxis and tracer
119 experiments with the cryptic *L. marina* species and other bacterivorous nematodes have
120 shown that they can selectively migrate towards and/or feed on bacterial strains (Derycke S.,
121 personal observations; Estifanos *et al.* 2013; Moens *et al.* 1999). If such selective feeding is
122 present in sympatrically distributed cryptic nematode species, this would support the idea that
123 niche partitioning is an important process allowing their coexistence. Bacteria are the main
124 food source of *Litoditis marina*, but occasionally also small green algae are taken up (Moens
125 & Vincx 1997). As such, *L. marina* is considered to be a deposit feeder (Moens & Vincx
126 1997). The oesophagus contains a distinct middle bulb and a poorly developed posterior bulb
127 with valves (Inglis & Coles 1961) which is very similar to the oesphagus of *C. elegans* and

which grinds the bacteria before transmission to the intestine (Seymour *et al.* 1983). The microbiome 'sensu lato' may thus also be linked to feeding behavior.

The aim of this study was to characterize the bacterial communities associated with co-occurring cryptic nematode species to reveal the extent of intra- and interspecific differentiation in the microbiome under natural field conditions. Single nematode specimens from each of three co-occurring species were simultaneously isolated from the same habitat in the same location, and a fragment of the microbial 16S rRNA gene was sequenced using the 454 GS FLX system (Roche). Next, to test whether the observed differences in bacterial communities are linked to resource use, we conducted a laboratory experiment with Pm1 and Pm3 nematodes which had been starved for two days before offering them *Escherichia coli* or a diverse bacterial mix. We expected to find significant differences in OTU composition between the two food treatments if the bacterial communities detected with the NGS approach indeed reflect resource use. Moreover, significant differences between species irrespective of food would indicate the presence of species specific microbiomes, which may help explain their differences in abiotic tolerances (Cabreiro & Gems 2013).

(b)Material and methods

Specimen collection

Individual nematode specimens have been collected in the framework of a geographical and seasonal investigation of the population genetic diversity in coastal and estuarine environments in Belgium and the southwest of The Netherlands in 2003 (Derycke *et al.* 2006). This study revealed that three closely related, cryptic *Litoditis* species (at that time *Pellioditis* marina) were co-occurring in the Paulina saltmarsh (51°21'N, 3°49'E) in October 2003 (Appendix S1). Fragments of living *Fucus* sp., one of the preferred habitats for *L.*

marina, were randomly collected and incubated on agar slants (Moens & Vincx, 1998). Nematodes were subsequently allowed to colonize the agar for about two days, during which they were able to feed on the natural bacteria associated with the *Fucus* fragments. No *E. coli* was added to these agarslants. After two days, specimens belonging to the *L. marina* speciescomplex were identified under a dissecting microscope using diagnostic morphological characters (Inglis & Coles 1961) and handpicked from the agar with a fine needle. All worms were digitally photographed using light microscopy, and stored individually in 70 – 95 % acetone until processed. Specimens were then assigned to cryptic species based on the COI genotyping from the population genetic survey (Derycke *et al.* 2006). We randomly selected six nematode specimens each of Pm1, Pm2 and Pm3 from the Paulina marsh samples.

DNA extraction and nematode identification

DNA was extracted using a simple lysis procedure by transferring individual nematodes to Worm Lysis Buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45 % NP40, 0.45 % Tween20). The worms were then cut in pieces with a razor blade, frozen for 10 min at -20 °C and subjected to proteinase K (60 µg/ml) treatment. Finally, the DNA samples were centrifuged for 1 min at maximum speed (13200 rpm) and the supernatant was used in the subsequent PCR. In the original study, the mitochondrial cytochrome oxidase c subunit 1 (COI) gene was amplified and analysed using Single Strand Conformation Polymorphism (Derycke *et al.* 2006). To double-check species identity, we re-amplified and sequenced the COI gene of all specimens for which we still had sufficient DNA. PCR amplification was done in 25 µl PCR reactions for 35 cycles, each consisting of a 30 s denaturation at 94 °C, 30 s annealing at 50 °C, and 30 s extension at 72 °C, with an initial denaturation step of 5 min at 94 °C and a final extension step of 5 min at 72 °C. Primers JB3 and JB5 were used (Derycke

175 *et al.* 2006) and unidirectional Sanger sequencing was done with JB3 by Macrogen. The
176 obtained sequences were then compared to published sequences of the *Litoditis marina*
177 species complex (Derycke *et al.* 2008a). All samples used in this study had COI sequences
178 that matched the SSCP based identification.

179 *16S rRNA gene amplification and 454 GS FLX sequencing of individual nematode specimens*
180 *from the field*

181 The bacterial communities associated with the six specimens from each of the three co-
182 occurring nematode species Pm1, Pm2 and Pm3 were characterized through amplification of
183 a portion of the 16S rRNA gene using the DNA extracts from the previous study. The 16S
184 rRNA gene was amplified using primers 968F and 1401R (Zoetendal *et al.* 1998).
185 Amplification was done in 50µl reactions containing 37.3 µl water, 5 µl buffer (10X), 1 µl
186 dNTPs (10mM each), 2 µl of each primer (10 µM) 0.2 µl Toptaq polymerase (Qiagen) and 2.5
187 µl DNA. Cycling conditions consisted of an initial denaturation of 2 min at 95°C, followed by
188 35 cycles of 95°C for 1 min, 53°C for 45s, 72°C for 3 min, and a final extension of 72°C for
189 10 min. The number of cycles follows that of other environmental bacterial surveys
190 (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). The forward primer
191 contained the Roche A adaptor (CGTATCGCCTCCCTCGCGCCATCAG) and an 11 bp MID
192 tag, while the reverse primer contained the Roche B adaptor
193 (CTATGCGCCTTGCCAGCCCGCTCAG) and an 11 bp MID tag. The MID tags are
194 provided in Appendix S2 and allowed separation of the sequences according to the nine
195 nematode specimens. The resulting fragment was 505 bp long. A 'no template' control was
196 included for each primer set to ensure no contamination occurred in the lab. PCR products
197 were checked on 1% agarose gels, purified with AMPure beads following the manufacturer's
198 protocol (Beckman Coulter Inc.), and measured with a Qubit fluorometer (Life technologies).

Samples were then pooled in equimolar concentrations and loaded on the Bioanalyzer (Agilent Technologies) to check the presence of a single peak. The pooled sample was bidirectionally sequenced on 1/8 of a 454 GS FLX plate (Macrogen). Two runs were performed, each containing three specimens from each species.

Data analysis

The raw datasets from the two runs were filtered and denoised with FlowClus (Gaspar & Thomas 2015), a program that uses the flow information in the sff.file to screen and correct errors. FlowClus is available for downloading at http://sourceforge.net/projects/flowclus/. Primers and barcodes were removed from the sequences and the reverse complement was taken of the reverse sequences. Filtering involved removal of sequences that were outside the 200 - 1000 bp range, had an average quality less than 25, or contained more than six homopolymers. Denoising was chosen with a constant value of 0.5. Chimera's were detected using Uchime without reference database (Edgar *et al.* 2011) and removed from the dataset. The sequences were then processed using QIIME 1.9.0 (Caporaso *et al.* 2010). Forward sequences from both runs were merged to create a dataset with only forward sequences. The reverse sequences from both runs were also merged to create a dataset with only reverse sequences. Unlike for the paired-end reads generated with Illumina, the forward and reverse datasets generated by the 454 protocol cannot be merged because forward and reverse reads are not generated from the same PCR molecule. Therefore, the resulting forward and reverse datasets were independently clustered into OTUs with 97% similarity using an open-reference OTU picking strategy. OTUs that were only observed once in the total dataset were removed because these are most likely to represent sequencing errors or rare variants within genomes. Default settings of QIIME 1.9.0 were used, except for the subsampling in the open reference OTU picking strategy, which was set at 0.01 instead of 0.001. The number of sequences and

OTUs obtained for each of the 18 specimens is summarized in Appendix S3 in Supporting Information.

Taxonomy was assigned up to species level using the assign_taxonomy.py script and the 97% taxonomy and OTU files of the Greengenes 13.8 database, using the default settings of the Uclust algorithm as implemented in QIIME. When no hit was observed, OTUs were labeled as 'Unassigned'. The taxonomic compositions associated with each of the three nematode species were visualized through bar graphs in excel using the unrarefied dataset for both F and R datasets.

Diversity within and between the three cryptic species was compared. To account for differences in number of sequences for each specimen, the dataset was rarefied at 600 sequences per specimen for each dataset. This number was slightly lower than the lowest number observed in our samples (626 for the Forward dataset, 643 for the Reverse dataset, see Appendix S1). Alpha diversity (Shannon Wiener, observed OTUs, Good's coverage) was calculated using alpha_rarefaction.py in QIIME. Rank abundance graphs were constructed to explore the abundance of OTUs associated with each nematode specimen. Generalized UniFrac distances ($\alpha = 0.05$) (Chen *et al.* 2012) were calculated with the GUnifrac package in R (Team 2008). Permanova was conducted on these UniFrac distances with species as grouping variable using the Adonis package in R. Permdisp and pairwise difference tests were also performed in R. Principal coordinates analysis (PCoA) plots were generated to visualize intra- and interspecific differences between the treatments using the Ade4 package in R. In addition, we investigated whether differences between species were caused by differences in rare OTUs, by constructing a dataset with only those OTUs that had at least 108 sequences (i.e. 1% of the rarefied dataset, which contained 18*600= 10800 sequences). This resulted in a

forward and reverse dataset containing 18 OTUs with a frequency higher than 1%. Statistical analyses on these datasets were perfomed as described above.

To investigate whether each of the nematode species had bacterial OTUs that were present in all specimens of that particular species (= the core microbiome of each species), we ran the compute_core_microbiome.py script. The frequency of the core OTUs in each specimen was visualized using the sequence counts from the rarefied biom table. Because many bacterial strains show a lower than 3% divergence, we investigated whether the core community would be impacted by clustering OTUs at 99% instead of 97%. For this, we reran the open-reference OTU picking strategy for the reverse dataset using a similarity of 99%. Taxonomic assignment was done using the 99% taxonomy and OTU files of the Greengenes 13.8 database. All other settings and parameters and core microbiome analysis were identical as mentioned above.

Biomarker taxa that are most likely to explain differences in microbiome between the three nematode species were assessed using the Linear discrimant analysis Effect Size (Segata *et al.* 2011) module as implemented in Galaxy (https://huttenhower.sph.harvard.edu/galaxy). Default settings were used, and species were selected as Class and specimens as subjects. We used the rarefied reverse dataset clustered at 97%.

Food experiment

To investigate whether the bacterial communities associated with the nematodes were part of the diet, living worms of Pm1 and Pm3 were subjected to two different food treatments: an *E.coli* treatment (Pm1E and Pm3E) and a 'bacterial mixture' treatment (Pm1B and Pm3B) in which nematodes were fed a natural inoculum of bacteria from the field. Fragments of the seaweed *Fucus* sp. from Paulina were put in culture flasks with artificial seawater (ASW)

269 with a salinity of 25 for one week at a temperature of 15°C and afterwards rinsed in ASW
270 with a salinity of 25. The ASW from the culture flasks and the washing step was filtered three
271 times over a GF/C filter with a diameter of 1.2 µm to remove organisms with sizes exceeding
272 those of bacteria, and frozen at -20°C until the experiment started. Two times 5 µL of this
273 suspension was used for DNA extraction for later bacterial diversity analysis ('bacterial
274 mixture'). Four Petri dishes of 5 cm inner diameter were filled with 4 mL of 1% bacto agar
275 medium (salinity of 25 and buffered at a pH of 7.5 – 8 with TRIS-HCl in a final concentration
276 of 5mM). Two dishes received 50 µL of a suspension of frozen-and-thawed *E. coli* (strain
277 K12 in PBS buffer) with a density of $3x10^9$ cells ml^{-1} to which either 20 adult Pm1 or 20 adult
278 Pm3 nematodes were added. The two remaining dishes received 50 µL of the bacterial mix
279 prepared from the *Fucus* thalli to which either 20 adult Pm1 or Pm3 nematodes were added.
280 Monospecific cultures of the two cryptic species were raised from one single gravid female
281 per species collected from Paulina marsh (The Netherlands) in March 2014 and maintained on
282 sloppy (0.8%) nutrient:bacto agar media (temperature of 20°C; salinity of 25) with
283 unidentified bacteria from their habitat as food (Moens & Vincx 1998). Two pieces of agar of
284 each nematode culture (Pm1 and Pm3) were subjected to a DNA extraction and 16S rRNA
285 gene amplification to pinpoint the bacteria that are able to grow on the culture medium.
286 Nematodes were allowed to feed on the bacteria for two days, after which ten nematodes per
287 treatment were picked out and quickly washed in cold sterile ASW to remove most of the
288 adherent bacteria. Subsequently, they were put individually in 20µL WLB for DNA
289 extraction. The DNA extraction was the same as described for the field specimens. For the
290 pure bacterial mixture a DNA clean-up (Wizard) was necessary after the DNA extraction, due
291 to the high salt concentration in the solution. In total, 46 DNA extracts were prepared (10 for

each of the four food treatments, 2 from the agar from each stock culture, and 2 from the bacterial mixture).

16S rRNA amplification and Illumina MiSeq sequencing of individual nematode specimens from the food experiment

For the DNA amplification and Illumina MiSeq sequencing a slightly adapted version of the protocol of the Earth Microbiome Project (Gilbert *et al.* 2014) was used. Amplification was done in 20µl reactions containing 11.4 µl water, 4µl 10X buffer, 0.4µl dNTP's (10 mM), 0.2µl Phusion (high fidelity) polymerase, 2µl DNA template and l µL forward and 1µl reverse primer (both 10µM). The forward primer contained the 5' Illumina adaptor, forward primer pad and linker and the 515f primer. The reverse primer consisted of the reverse complement of the 3' Illumina adapter, the reverse primer pad and linker, the 806r primer and a Golay barcode. This Golay barcode was unique for each sample and the first 52 barcodes of the Earth Microbiome Project were used (Caporaso *et al.* 2012). Cycling conditions consisted of an initial denaturation of 30s at 98°C, followed by 35 cycles of 98°C for 10s, 65°C for 30s, 72°C for15s, and a final extension of 72°C for 10 min. Samples were amplified in triplicates. Three samples were randomly chosen in which the triplicates received different barcodes to allow investigation of PCR cycle bias. We did detect some PCR bias, but most OTUs were shared between replica's and OTUs uniquely found in one replica reached only very low frequencies (maximum of 0.21%). All analyses regarding the technical replicates can be found in Appendix S43. After amplification, triplicates were combined. PCR products were cleaned by selecting the correctly sized bands (300 – 350 bp) with the help of Clone-Well Agarose Gels (E-Gel). After this, the PCR concentration was measured with the Qubit Fluorometer (Life Technologies) and an equal amount of amplicon from each sample was pooled into one single, sterile tube. The final sample was checked for concentration and

quality with the BioAnalyzer (Agilent Technologies). Illumina MiSeq sequencing was performed by the Genomics Core (UZ Leuven). Because only a small amount of reads from the nematodes fed *E. coli* were assigned to Enterobacteraceae (see results), the *E. coli* suspension was sequenced in a separate MiSeqrun (as part of a follow-up experiment) to exclude any methodological issues. Three biological replica's of the suspension were amplified and sequenced as described above.

Data analysis

The Illumina paired-end sequences were first assembled with PEAR (Paired-end reader merger (Zhang *et al.* 2014)). Subsequent filtering involved trimming of reads with a quality score of 25, read lengths had to be in the 200 -1000 bp range, and all reads containing uncalled bases were discarded. Subsequently, forward and reverse primers were removed with Cutadapt (Martin 2011). The sequences were then processed using QIIME 1.8.0 (Caporaso *et al.* 2010) with an open reference OTU picking strategy (97% clustering) as described above. Beta diversity analyses involved rarefaction of the dataset at 41000 sequences for each sample. Generalized UniFrac distances ($\alpha = 0.05$) (Chen *et al.* 2012) and statistical analyses were calculated in R as described above. The technical replicates that received a different barcode to investigate PCR bias were merged into a single sample for alpha and beta diversity analyses. The rarefied dataset was also used to identify biomarker taxa between Pm1 and Pm3 related to resource use using species as class, food treatment as subclass and specimens as subjects. Default settings were used.

The *E. coli* samples were separately analysed from the first MiSeq run, but the same assemblage, filtering, trimming, OTU clustering and taxonomic assignment procedures were used.

Scanning Electron Microscopy (SEM)

In our previous study, all specimens were photographed digitally prior to the DNA extraction to have a morphological reference before being stored in acetone. To assess the abundance of bacteria associated with the nematode cuticle, we reexamined the digital pictures of the specimens used for next generation sequencing. In addition, nematodes grown on agar media with unidentified bacteria from their habitat and *E. coli* as additional food, from monospecific cultures of each of the three nematode species were used to generate SEM pictures of the head, tail and midbody region. These SEM pictures were generated to investigate the abundance and diversity of bacteria on the cuticle of the nematodes. The numbers of females photographed were 7, 3 and 7 for Pm1, Pm2 and Pm3 respectively, and the numbers of males were 9, 4 and 3, respectively. SEM pictures were generated with the JEOL JSM-840 scanning electron microscope by the Nematology Unit of the Biology Department at Ghent University.

(c) Results

16S rRNA composition of individual nematode specimens from the field

Taxonomic composition of the bacterial communities associated with cryptic species

Taxonomic assignments at the phylum level were highly comparable for the Forward and Reverse datasets and only differed in the presence of an additional three 'phyla' ('unidentified bacteria', Planctomycetes and 'TM6') in very low frequency in the Reverse dataset. We restrict the detailed description of the taxonomic composition to the Reverse dataset, because it yielded slightly more sequences for each sample (Appendix S3). Taxonomic composition at the phylum level for the forward dataset can be found in Appendix S5.

The microbiomes of all three nematode species were dominated by the phylum Proteobacteria (53%, 70% and 73% for Pm1, Pm2 and Pm3, respectively). The phyla Bacteroidetes (10%, 14% and 1.8% for Pm1, Pm2 and Pm3, respectively) and Actinobacteria (17%, 6% and 5% for Pm1, Pm2 and Pm3, respectively) were the second and third most abundant group of bacteria, which were found in nearly all specimens (17 and 18 of the specimens, respectively). The Verrucomicrobia were present in 5 of the 6 specimens of Pm3 with an average relative frequency of 16%, whereas its frequency in Pm1 and Pm2 was less than 1% and 4%, respectively and in 4 and 2 of the 6 specimens, respectively. The Firmicutes group was present in all 18 specimens in similar frequencies (2.1%, 3.6% and 3.8% in species Pm1, Pm2 and Pm3 respectively). In total, 79 OTUs were unassigned, but nearly all of them had a relative frequency of less than 1% and their total abundance reached 9.9%. New.ReferenceOTU30 was prominent in Pm1 (12 % in the rarefied dataset), but only in one replicate. Within the phylum Proteobacteria, the Gammaproteobacteria dominated the microbiomes of Pm1 (82.7%) and Pm2 (72.7%) and to a lesser extent the microbiome of Pm3

374 (46.4%) (Fig 1A) and contained 57 taxa from 22 known families (Fig 1B). The
375 Alteromonadaceae and Moraxellaceae were amongst the most abundant families shared
376 between the three species and were especially abundant in Pm3 (12.6% and 15.5%) (Fig1B).
377 The Alphaproteobacteria formed the second most abundant class within the Proteobacteria,
378 and represented 9.6%, 18.1% and 44.2% of the assigned taxa of Pm1, Pm2 and Pm3,
379 respectively (Fig 1A). This group comprised 44 taxa belonging to 15 known families, of
380 which the Caulobacteraceae, Rhodobacteraceae and Sphingomonadaceae were the most
381 abundant (Fig 1C). Especially the latter family was much more abundant in Pm3 (20.7%) than
382 in Pm1 (1.8 %) and Pm2 (1.7%) but this was caused by a high abundance in one specimen
383 (175Pm3, Fig 1C). The Beta, Delta and Epsilon Proteobacteria were only poorly represented,
384 and contained 28, 10 and 2 taxa, respectively.

385 Within the phylum Actinobacteria, more than 99% of the taxa belonged to the Actinobacteria
386 class, within which 17 families were assigned (Fig 2A). Two families, the Corynebacteriaceae
387 and the Microbacteriaceae, were prominent in all three nematode species. The high abundance
388 of the Microbacteriaceae in Pm1 was mainly caused by a high abundance in a single specimen
389 (145Pm1, Fig 2A).

390 Within the phylum Bacteroidetes, two classes encompassed more than 99% of the assigned
391 taxa: the Flavobacteria dominated Pm1 and Pm3 (75.3% and 81.8%, respectively), while the
392 Cytophagia dominated Pm2 (68.0%, versus 23.2% and 17.4% in Pm1 and Pm3). Both classes
393 were represented by only two families: the Cytophagia consisted of Cytophagaceae and
394 Flammeavirgaceae (Fig 2B), the latter being found in very low abundance and in only one
395 specimen of each species; the Flavobacteria consisted of Flavobacteriaceae, Cryomorphaceae
396 and Weeksellaceae, the former being dominant in Pm1, while the Weeksellaceae were
397 abundant in Pm2 (Fig 2B).

Alpha diversity of field specimens

Rarefaction curves of the number of observed OTUs yielded highly similar results for Forward and Reverse datasets. Curves were still increasing at a sampling depth of 600 sequences per nematode specimen (Fig 3a, appendix S5). In contrast, the Shannon diversity measure quickly reached a plateau (Fig 3b, appendix S5), suggesting that many OTUs occur in very low frequencies. This was confirmed by the rank abundance plot, which illustrates that only a few OTUs have relative abundances higher than 0.1, while many OTUs have very low relative abundances (Appendix S6).

Beta diversity of field specimens

Permanova based on the Generalized Unifrac distances showed significant differences between the microbial communities of the nematode species for both Forward and Reverse datasets and with or without inclusion of rare OTUs (Table 1). Post hoc tests revealed that these differences were situated between Pm1 and Pm3, regardless of the dataset used. The six specimens within species did, however, show substantial variability (Fig 4, appendix S5). The non-significant PERMDISP results (Table 1) indicated that intraspecific differences were comparable for each of the three species.

The core microbiome of field specimens

Despite the high number of OTUs observed for each nematode species (see Appendix S3), none of them were shared between all 18 specimens. The core microbial community for each species consisted of very few OTUs (5, 5 and 2 OTUs for species Pm1, Pm2 and Pm3 in the Forward dataset, respectively, and 5, 6 and 4 OTUs for species Pm1, Pm2 and Pm3 in the Reverse dataset, respectively; see Appendix S7). Frequencies of the core communities were overall low in each of the 18 specimens, but 4 and 6 core OTUs of the forward and reverse

datasets respectively reached frequencies higher than 1% (Fig 5). The core communities of species Pm1 and Pm2 were also present in the other species, while the core community of species Pm3 was nearly absent in the two other species. Permanova on the generalized unifrac distances yielded only borderline (non) significant differences between the three species (Reverse dataset: $F = 2.40$, $p = 0.058$; Forward dataset: $F = 2.94$, $p = 0.048$), suggesting that the core communities were phylogenetically similar to each other. Small differences in taxonomic composition were however present (Appendix S7). OTU clustering at 99% slightly increased the number of core OTUs (8 vs 5 for Pm1, 6 vs 6 for Pm2 and 5 vs 4 for Pm3) which was mainly due to an increase of OTUs identified as Moraxellaceae. Taxonomic composition was very similar to that observed with 97% clustering (Appendix S7).

Biomarker taxa of the field specimens

The LeFSe analysis indicated 1, 2 and 6 taxa that significantly differentiated Pm1, Pm2 and Pm3 respectively and with an LDA score higher than two. The biomarker for Pm1 belongs to the genus *Pseudoalteromonas* (OTU4406967). New.ReferenceOTU37 and OTU200979 were identified as biomarker for Pm2 and belong to the genus *Microbacterium* and the ordo Saprospirales respectively. The biomarker taxa of Pm3 were identified as Verrucomicrobiaceae (New.ReferenceOTU54 and OTU4307243), *Acinetobacter* (OTU4449456), Moraxellaceae (OTU4334053), Caulobacteraceae (OTU310003) and Comamonadaceae (OTU115161) (Appendix S7).

16S rRNA composition of individual nematode specimens from the food experiment

To investigate whether the observed differences in the microbiomes of Pm1 and Pm3 were related to selective feeding, we performed a food experiment in which both species were

offered *E. coli* or a diverse bacterial mix as food. The MiSeq protocol generated a much larger number of sequences and OTUs per nematode specimen (Appendix S8) than the 454 protocol. A detailed description of the taxonomic composition of the non-rarefied dataset of the food experiment can be found in Appendix S9. The microbiomes of all samples were clearly dominated by Proteobacteria and Bacteroidetes (Fig 1 in Appendix S9). At the family level, the microbiomes of the two food treatments showed some striking differences between each other, but also between species: 1/ within Alphaproteobacteria, the microbiomes of Pm3 worms fed the bacterial mixture resembled the bacterial mixture, while the microbiomes of the Pm3 worms fed *E. coli* contained a substantial amount of Rhodobacteraceae, which were highly abundant in the Pm3 stock cultures (Fig 6A). In contrast, Pm1 worms showed very similar compositions regardless of the offered food. 2/ Within Gammaproteobacteria, the microbiomes of Pm1 and Pm3 fed the bacterial mix were similar to that of the bacterial mix. The microbiomes of Pm1 and Pm3 worms fed *E. coli* resembled that of the stock cultures of each species (Fig 6A). Surprisingly, the worms fed *E. coli* ~~treatments~~ were not enriched for Enterobacteriaceae. However, the *E. coli* suspension that was offered to the nematodes in the *E. coli* treatments was dominated by Enterobacteraceae (Fig 6B). 3/ Within the Bacteroidetes, all Pm3 worms were dominated by Saprospiraceae, the dominant family of the bacterial mix. Abundances of this family were higher in the Pm3 worms fed the bacterial mix than those that had been fed *E. coli*. For Pm1, taxonomic composition of both food treatments was comparable (Fig 6A).

Alpha diversity of specimens from the food experiment

The average number of OTUs observed in the nematodes fed the bacterial mix was similar to that in those fed *E. coli* (Kruskall-Wallis: df = 6, p = 0.08). Patterns of species diversity and richness were very similar to the data on the field specimens: the number of OTUs was still

increasing at a sampling depth of 41000 sequences per treatment, the Shannon diversity measure quickly reached a plateau, and the rank abundance plots again show that many OTUs have very low relative abundances (Appendix S9). Four OTUs were highly abundant in the Pm1 specimens from the *E. coli* treatment and are thus likely to be part of the microbiome sensu stricto: *Pseudoalteromonas* (ca 98 000 reads), *Agrobacterium* (ca 69 000 reads), Unassigned (ca 57 000 reads) and *Winogradskyella thalassocola* (ca 32 000 reads). When blasted in Genbank, the unidentified OTU was most similar to an uncultured bacteria from a water cave (accession number FJ604748.1). The most highly abundant Pm3E OTU (ca 150 000 reads) was the same unidentified OTU as for Pm1E.

Beta diversity of specimens from the food experiment

Permanova based on the Generalized UniFrac distances of the four food x species treatments (Pm1B, Pm1E, Pm3B, Pm3E) showed significant differences between food (pseudo $F_{1,39}$= 3.42; p=0.005) and species (pseudo $F_{1,39}$= 10.97; p=0.001). The interaction between food and species was only just significant (pseudo $F_{1,39}$= 2.02; p=0.049). Pairwise comparisons were all significant, except for Pm1B and Pm1E (Table 2). The principal coordinates analysis showed that species is the most important grouping factor (Fig 7). Within each species, Pm1 showed high intraspecific variability in both food treatments, while intraspecific variability for Pm3 was much lower in the treatment where they were offered a bacterial mix. Homogeneity of dispersions was not achieved (p>0.05) for factor species, reflecting the high variation within Pm1.

The core microbiome of specimens from the food experiment

Similar to the results of the field specimens, the fraction of OTUs shared between all specimens was very low. In total, 41 OTUs were shared between all 46 samples of the food

492 experiment. The core of the Pm1 bacterial mixture treatment had 157 OTUs and the Pm3
493 bacterial mixture treatment had 261 core OTUs. The number of core OTUs was lower for the
494 *E.coli* treatment: 85 core OTUs were present in Pm1 and 178 for Pm3. The core of all 20 Pm3
495 individuals contained 77 OTUs, while Pm1 had 52 OTUs shared among all 20 specimens.
496 Permanova on UniFrac distances showed that food (pseudo-$F_{1,39}$=3.59,p=0.008), species
497 (pseudo-$F_{1,39}$=16.56, p=0.001) and the interaction food*species (pseudo-$F_{1,39}$=2.46,=0.043)
498 were significant. All pairwise comparisons were significant, except for the two food
499 treatments of Pm1 (Table 2).

500 *Biomarker taxa of specimens from the food experiment*

501 For Pm1, 433 OTUs were identified as biomarkers, while 208 OTUs were identified as
502 biomarker for Pm3. Taxonomic assignment of many OTUs was only achieved at the class
503 level and 52 OTUs of the Pm3 biomarker taxa had no taxonomic assignment at all
504 (Appendix S11). The biomarker OTUs that were identified up to family level belonged to the
505 Flavobacteriaceae, Rhodobacteraceae, Alteromonadaceae, Pseudoalteromonadaceae and
506 Vibrionaceae for both species, with an additional two families for the biomarker taxa of Pm1
507 (Phyllobacteriaceae and one unidentified family of the ordo Saprospirales). The complete list
508 of biomarker OTUs for Pm1 and Pm3 with their taxonomic assignment can be found in
509 Appendix S11.

510 *SEM and light microscope pictures*

511 SEM pictures revealed that the cuticle of the cryptic nematode species contained only very
512 few bacteria, which were mainly located in the mid body region for the females, and in the
513 tail region for the males (see Appendix S12). The morphology of the attached bacteria was
514 quite uniform, suggesting a very low taxonomic diversity of the epibionts. The digital pictures

515 that were taken from the sequenced specimens seconds before transferring them into the WLB
516 further support that the bacterial densities and diversity on the cuticle of the three rhabditid
517 nematodes were low.

518 (d) Discussion

519 *The nematode microbiome is highly diverse and species specific*

520 Our data show that the bacterial community associated with the *Litoditis* specimens contains
521 at least 85 OTUs for the field specimens (Appendix S3). Most OTUs were present in very low
522 frequency. Even under laboratory conditions and with *E. coli* as a food source, a high
523 diversity was associated with the nematode specimens (lowest number: 1118 OTUs,
524 Appendix S8). Applying the MiSeq protocol to the field specimens would very likely result in
525 an even higher diversity than observed in the laboratory specimens. The microbiomes of the
526 field specimens and cultured nematodes are not directly comparable because two different
527 sequencing platforms (454 vs. Illumina platforms) and primer sets were used to generate
528 sequence data which may introduce taxonomic and technical biases in terms of the microbial
529 community recovered.

530 Despite the high number of bacterial OTUs associated with the field nematode specimens,
531 only 2 - 6 OTUs were found in all six specimens of a particular species, and not a single OTU
532 was found in all 18 specimens (see Appendix S7). This was also true for the food experiment,
533 in which 52 OTUs were shared among the 20 Pm1 specimens and 77 OTUs were shared
534 amongst the 20 Pm3 specimens. The frequency of the core microbiome was very low, and
535 although six core OTUs obtained a frequency higher than 1% in the rarefied dataset, their
536 abundance varied substantially between individuals (Figure 5). Bacterial strains that are
537 present in the core microbiome of a particular nematode species and that are absent in the

other species can potentially confer an adaptation to the environment for that particular nematode species. Moreover, if such core OTUs are also present in the other nematode species than the species for which it is a core OTU, its abundance should be significantly different between nematode species. In other words, it would be identified as biomarker in the LeFSe analysis. Three core OTUs of Pm3 were completely absent in Pm1 when clustering at 97% for the reverse dataset (appendix S7): OTU310003 (Caulobacteraceae), OTU720489 (*Acinetobacter*) and OTU4449456 (*Acinetobacter*). They may thus be involved in mediating different tolerances to environmental conditions for Pm1 and Pm3. Two of these Pm3 core OTUs were also present in Pm2 (OTU310003 and OTU4449456) and were identified as biomarkers for Pm3 by the LeFSe analysis, suggesting that members of Caulobacteraceae and *Acinetobacter* may be involved in differential abiotic tolerances for Pm3. All Pm2 core OTUs were present in the two other species, and only one was identified as a biomarker for Pm2: OTU200979 (*Microbacterium*). This OTU may thus potentially be involved in generating tolerance to abiotic conditions for Pm2. Laboratory experiments show that Pm1 performs less well at higher temperatures, while population development of Pm3 was lower at lower temperatures (De Meester *et al.* 2015b). This corresponds with the prevalence of Pm3 during warmer seasons and to its near-absence during colder seasons (Derycke *et al.* 2006). Pm2 has a pan European distribution and appears to be a generalist as it is found in habitats that differ substantially in temperature and salinity (Derycke *et al.* 2008b). The microbiome '*sensu stricto*' may perform a critical role in the physiological adaptations to such environmental changes.

Sympatric, cryptic nematode species show differences in resource use

We hypothesized that the differences in the microbiomes '*sensu lato'* between the nematode species were linked to differential resource use, as all three species are bacterivorous. We

562 expected to find many more OTUs in the worms that had been feeding on the bacterial mix
563 compared to those that had been fed *E. coli*. This appeared not to be the case, but there was a
564 significant food effect (Table 2) on the microbiome, indicating that bacteria were
565 differentially consumed by the worms in the two food treatments. The similar number of
566 OTUs observed in both food treatments may indicate that the worms only fed on a small
567 number of OTUs present in the bacterial mix. Yet, the taxonomic composition of the worms
568 fed on the bacterial mix was quite diverse and resembled the one of the bacterial mix. The
569 stock cultures of both worms contained a large number of OTUs (1996 and 1301 for Pm1 and
570 Pm3 respectively, appendix S8) indicating that the microbiome *sensu stricto* is highly diverse
571 and that several bacterial strains of this microbiome are able to grow on the agar. The Pm1
572 and Pm3 microbiomes from the *E. coli* treatment shared 1271 and 1135 OTUs with the Pm1
573 and Pm3 culture microbiome, respectively. Consequently, the potential food of the worms in
574 the *E. coli* treatment was probably as diverse as the bacterial mix (which contained 2496
575 OTUs versus 552 OTUs for the *E. coli* suspension, appendix S8). OTUs showing higher
576 abundances in the cultures did not result in a higher abundance in the microbiome and vice
577 versa. Moreover, the microbiomes of specimens fed with *E. coli* resembled the one of the
578 stock cultures, and their intestinal colour clearly indicated that they were actively feeding to a
579 similar extent as the specimens in the bacterial mix treatment, adding support to the idea that
580 the worms in the *E. coli* treatments had a much more diverse food source than anticipated.
581 Surprisingly, we did not find an increase of Enterobacteraceae in the specimens fed *E. coli*.
582 Yet, the *E. coli* suspension was clearly dominated by Enterobacteraceae (Fig 6B), providing
583 evidence that our methodological approach was able to identify the *E. coli* sequences. The *E.*
584 *coli* source consisted of frozen and thawed *E. coli* cells, and provided as such a “soup” rich of
585 nutrients instead of metabolically active cells. Add-back experiments have demonstrated that

C. elegans requires metabolically active cells for normal development and fecundity (Lenaerts *et al.* 2008). Tracer experiments with *Litoditis* showed that radioactive labels were only present in the worms when fed labeled (unidentified) bacteria, while such a radioactive signal was absent when the worms were offered the growth medium of that same bacterial mix without cells despite the fact that this medium was much more heavily labeled than the bacterial cells (Moens, unpublished data). This suggests that the nutrient rich "soup" provided by the *E. coli* suspension can stimulate extensive growth of other bacteria from the worm microbiome and that the soup itself was not ingested by the worms.

The food experiment further showed that the microbiome of Pm1 did not differ according to food type, while that of Pm3 did. This result can be explained by two non-mutually exclusive scenarios: 1/ the Pm3 microbiome '*sensu stricto*' (Pm3E) differs considerably from the bacterial mixture while the Pm1 microbiome '*sensu stricto*' (Pm1E) is similar to the bacterial mixture. Feeding of Pm3 on the bacterial mix would then lead to significant differences between Pm3E-Pm3B but not between Pm1E-Pm1B. Comparison of the number of OTUs shared between the *E. coli* fed specimens and the bacterial mix do not support this hypothesis, since Pm3 specimens typically show a higher number of shared OTUs with the bacterial mix than Pm1 specimens (Appendix S13); 2/ the two species show different feeding behaviors with Pm3 feeding more selectively on a smaller portion of the bacterial mixture, while Pm1 feeds on a much wider range of bacterial strains from the mixture. This hypothesis is supported by the larger variability between individual Pm1 specimens that were fed the bacterial mix compared to the much smaller interindividual variability in Pm3 (PCoA plot, Fig 7) and by the higher number of biomarker taxa identified in Pm1 compared to Pm3 (Appendix S11), indicating that Pm3 is a much more selective feeder than Pm1. We also found a significant species effect (Table 2), suggesting that Pm1 and Pm3 were feeding on

610 different bacterial species. Since the Pm1 and Pm3 nematodes from the food experiment have
611 been kept for several generations under controlled abiotic conditions, the biomarker taxa
612 revealed by the LeFSe analysis are likely linked to differential resource use of the two
613 species. The individual differences in bacterial diet cannot be linked to particular life stages or
614 certain ecological morphs since we only selected adult specimens for our population genetic
615 analysis (Derycke *et al.* 2006). Observations on the feeding behavior of living *Litoditis*
616 *marina* specimens showed that the size of the prey forms an important filter for ingestion
617 (Moens & Vincx 1997; Tietjen & Lee 1975), and the buccal cavity of Pm3 specimens is
618 smaller than that of Pm1 specimens (Derycke *et al.* 2008a) suggesting that size selection may
619 be one aspect contributing to differences in selectivity. We cannot exclude that the four OTUs
620 potentially involved in adaptation to abiotic conditions are linked to resource use, but
621 *Microbacterium* was present in all three nematode species, and also different *Acinetobacter*
622 OTUs were found in all three nematode species, suggesting that these types of bacterial
623 strains can be ingested by all three species and that size selection through feeding may not be
624 an important mechanism to explain the different abundances of these core OTUs. Instead, the
625 high variability among individuals implies that there are constraints in resource use that
626 prevent individuals from using the whole range of available resources. These constraints may
627 act at the individual level (e.g. uptake ability, morphology, behavior), but probably more so at
628 the population level, where high intraspecific competition can increase individual niche
629 specialization (Svanback & Bolnick 2007). Intraspecific competition has been observed in all
630 three species (De Meester *et al.* 2015a) and individual niche specialization can increase the
631 niche breadth of the total population (Bolnick *et al.* 2007). This agrees well with the high
632 diversity of the microbiomes observed in each of the three species, and can affect interspecific

interactions, since niche overlap between species is likely to increase with increased niche width.

Niche partitioning between cryptic species can partially explain their coexistence

The nematode microbiomes were dominated by Alpha- and Gammaproteobacteria, Bacteroidetes and Verrucomicrobia which are the dominant groups found on *Fucus vesiculosus* (Lachnit *et al.* 2011), the habitat from which the nematode specimens were isolated. The microbiomes of Pm1 and Pm3 from the field were clearly different from each other, and the food experiment shows that these differences are linked both to the feeding activity of the species but also to the presence of a nematode species specific microbiome. Pm1 and Pm3 specimens more often co-occur in the field than Pm1 with Pm2 or than Pm3 with Pm2 (Appendix S1). These data agree well with the ecological theory of resource partitioning, where species can coexist when they are using different resources (MacArthur & Levins 1967). However, if resource partitioning would be the only driver for coexistence of these cryptic species, we would expect to find Pm1 coexisting with Pm3 throughout the year, which is not the case (Derycke *et al.* 2006). Coexistence of species is also governed by their common responses to environmental changes (Chesson 2000; Leibold & McPeek 2006) and the microbiome may perform a critical role in the physiological adaptations to such environmental changes and hence in the fitness of the nematode hosts. Dedicated attempts (using repeated transfer of worms through mixtures of antibiotics and even incorporating antibiotics in the stock culture media for several subsequent generations) at removing bacteria other than the *E. coli* supplied as food failed (P. Gilarte, unpublished data), suggesting a tight association between nematodes and (components of) their microbiomes. Fitness differences imply that differential responses to abiotic environmental variability can also have stabilizing effects on the coexistence between cryptic nematode species. In addition to these

deterministic variations in environment, the ephemeral nature of the *Fucus* habitat on which the species live induces strong stochastic variability in the environment. The coexistence of Pm1 and Pm3 is therefore likely to be determined by both resource partitioning and differential responses to abiotic changes. Although microbiome differentiation was less straightforward between Pm1 and Pm2, phylogeographic data revealed that Pm2 has a more widespread distribution than the two other species, suggesting it has a broader 'abiotic' niche than the other species. The microbiome of Pm2 was also not differentiated from either of the two other species.

Methodological considerations

Our understanding of the degree of resource selectivity in nematode feeding behavior is generally very poor: several laboratory experiments have demonstrated a high capacity to select among even very similar food items (Moens *et al.* 1999), but reliable approaches to study such detailed resource selectivity under more natural conditions have been lacking. Moreover, stable isotope and other approaches which measure food absorption usually require pooling of individuals for a single analysis (Carman & Fry 2002). Our approach complements others, but provides a substantial advance compared to any previous work on resource utilization of free-living nematodes or other microscopic eukaryotes by characterizing the complete bacterial community of individual specimens of three nematode species. The marker gene survey approach used here allows to assess selective feeding behaviour of single nematode specimens, which has not been possible with methods widely used to assess resource use (e.g. stable isotope analysis). However, our results also show the presence of a highly diverse endosymbiont community that differs substantially among individuals. Our morphological investigation of the bacteria on the cuticula detected only few bacterial

morphotypes suggesting that most of the microbiome is located inside the body of the worm (see Appendix S12).

Conclusion

The natural bacterial communities of sympatrically distributed cryptic nematode species are highly diverse and show pronounced intraspecific diversity. The species specific microbiomes may play a role in the different tolerances of the nematode species to abiotic conditions. Importantly, the differences in selective feeding of morphologically similar nematode species may have a cascading effect on the microbial community and on the functioning of the whole decomposition system, as alterations in microbial communities can alter mineralization of organic matter (Nascimento *et al.* 2012). Consequently, cryptic diversity may have hitherto unpredicted consequences for biodiversity-ecosystem functioning relationships in the marine benthos

IV. Acknowledgements

We gratefully acknowledge John Gaspar for his help in denoising the 454 GS FLX sequence data, and Marjolein Couvreur for generating the SEM pictures. S.D. is funded by the Belgian Science Policy Office (Belspo). This research was financially supported through the F.W.O. projects 3G019209 and 3G038715, and by Ghent University through the BOF project 01GA1911W. We are thankful for the valuable comments of three anonymous referees which greatly improved a previous version of this manuscript.

V. References

Bickford D, Lohman DJ, Sodhi NS*, et al.* (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* **22**, 148-155.

Bolnick DI, Svanback R, Araujo MS, Persson L (2007) Comparative support for the niche variation hypothesis that more generalized populations also are more heterogeneous. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 10075-10079.

Cabreiro F, Gems D (2013) Worms need microbes too: microbiota, health and aging in Caenorhabditis elegans. *EMBO Mol Med* **5**, 1300-1310.

Caporaso JG, Kuczynski J, Stombaugh J*, et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**, 335-336.

Caporaso JG, Lauber CL, Walters WA*, et al.* (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *Isme Journal* **6**, 1621-1624.

Carman KR, Fry B (2002) Small-sample methods for delta C-13 and delta N-15 analysis of the diets of marsh meiofaunal species using natural-abundance and tracer-addition isotope techniques. *Marine Ecology Progress Series* **240**, 85-92.

Chen J, Bittinger K, Charlson ES*, et al.* (2012) Associating microbiome composition with environmental covariates using generalized UniFrac distances. *Bioinformatics* **28**, 2106-2113.

Cheng XY, Tian XL, Wang YS*, et al.* (2013) Metagenomic analysis of the pinewood nematode microbiome reveals a symbiotic relationship critical for xenobiotics degradation. *Sci Rep* **3**, 1869.

Chesson P (2000) Mechanisms of maintenance of species diversity. *Annual Review of Ecology and Systematics* **31**, 343-+.

de Leon GPP, Nadler SA (2010) What we don't recognize can hurt us: a plea for awareness about cryptic species. *Journal of Parasitology* **96**, 453-464.

De Meester N, Derycke S, Bonte D, Moens T (2011) Salinity effects on the coexistence of cryptic species: A case study on marine nematodes. *Marine Biology* **158**, 2717-2726.

De Meester N, Derycke S, Moens T (2012) Differences in time until dispersal between cryptic species of a marine nematode species complex. *PLoS ONE* **7**, e42674.

De Meester N, Derycke S, Rigaux A, Moens T (2015a) Active dispersal is differentially affected by inter- and intraspecific competition in closely related nematode species. *Oikos* **124**, 561-570.

De Meester N, Derycke S, Rigaux A, Moens T (2015b) Temperature and salinity induce differential responses in life histories of cryptic nematode species. *Journal of Experimental Marine Biology and Ecology* **472**, 54-62.

Derycke S, Backeljau T, Moens T (2013) Dispersal and gene flow in free-living marine nematodes. *Frontiers in Zoology* **10**.

Derycke S, Backeljau T, Vlaeminck C*, et al.* (2006) Seasonal dynamics of population genetic structure in cryptic taxa of the *Pellioditis marina* complex (Nematoda: Rhabditida). *Genetica* **128**, 307-321.

Derycke S, Fonseca G, Vierstraete A*, et al.* (2008a) Disentangling taxonomy within the *Rhabditis (Pellioditis) marina* (Nematoda, Rhabditidae) species complex using molecular and morphological tools. *Zoological Journal of the Linnean Society* **152**, 1-15.

Derycke S, Remerie T, Backeljau T*, et al.* (2008b) Phylogeography of the *Rhabditis (Pellioditis) marina* species complex: evidence for long-distance dispersal, and for range expansions and restricted gene flow in the northeast Atlantic. *Molecular Ecology* **17**, 3306-3322.

Derycke S, Remerie T, Vierstraete A*, et al.* (2005) Mitochondrial DNA variation and cryptic speciation within the free-living marine nematode *Pellioditis marina*. *Marine Ecology Progress Series* **300**, 91-103.

747 Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of
748 chimera detection. *Bioinformatics* **27**, 2194-2200.
749 Elmer KR, Bonett RM, Wake DB, Lougheed SC (2013) Early Miocene origin and cryptic diversification
750 of South American salamanders. *BMC Evolutionary Biology* **13**.
751 Estifanos TK, Traunspurger W, Peters L (2013) Selective feeding in nematodes: a stable isotope
752 analysis of bacteria and algae as food sources for free-living nematodes. *Nematology* **15**, 1-
753 13.
754 Fonseca G, Derycke S, Moens T (2008) Integrative taxonomy in two free-living nematode species
755 complexes. *Biological Journal of the Linnean Society* **94**, 737-753.
756 Fonseca VG, Carvalho GR, Sung W*, et al.* (2010) Second-generation environmental sequencing
757 unmasks marine metazoan biodiversity. *Nature Communications* **1**.
758 Gaspar JM, Thomas WK (2015) FlowClus: efficiently filtering and denoising pyrosequenced amplicons.
759 *Bmc Bioinformatics* **16**.
760 Gilbert JA, Jansson JK, Knight R (2014) The Earth Microbiome project: successes and aspirations. *BMC*
761 *biology* **12**, 69.
762 Gingold R, Ibarra-Obando SE, Rocha-Olivares A (2011) Spatial aggregation patterns of free-living
763 marine nematodes in contrasting sandy beach micro-habitats. *Journal of the Marine*
764 *Biological Association of the United Kingdom* **91**, 615-622.
765 Glasby CJ, Wei NWV, Gibb KS (2013) Cryptic species of Nereididae (Annelida : Polychaeta) on
766 Australian coral reefs. *Invertebrate Systematics* **27**, 245-264.
767 Heip C, Vincx M, Vranken G (1985) The ecology of marine nematodes. *Oceanography and Marine*
768 *Biology: an Annual Review* **23**, 399-489.
769 Inglis W, Coles J (1961) The species of *Rhabditis* (Nematoda) found in rotting seaweed on British
770 beaches. *Bulletin of the British Museum of Natural History (Zoology)* **7**, 320-333.
771 Knowlton N (1993) Sibling species in the sea. *Annual Review of Ecology and Systematics* **24**, 189-216.
772 Lachnit T, Meske D, Wahl M, Harder T, Schmitz R (2011) Epibacterial community patterns on marine
773 macroalgae are host-specific but temporally variable. *Environmental Microbiology* **13**, 655-
774 665.
775 Leibold MA, McPeek MA (2006) Coexistence of the niche and neutral perspectives in community
776 ecology. *Ecology* **87**, 1399-1410.
777 Lenaerts I, Walker G, Van Hoorebeke L, Gems D, Vanfleteren J (2008) Dietary restriction of
778 *Caenorhabditis elegans* by axenic culture reflects nutritional requirement for constituents
779 provided by metabolically active microbes. *Journals of Gerontology Series A Biological*
780 *Sciences and Medical Sciences* **63**, 242-252.
781 MacArthur R, Levins R (1967) The limiting similarity, convergence, and divergence of coexisting
782 species. *American Naturalist* **101**, 377-385.
783 Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads.
784 *EMBnet.journal* **17**.
785 Moens T, Verbeeck L, de Maeyer A, Swings J, Vincx M (1999) Selective attraction of marine
786 bacterivorous nematodes to their bacterial food. *Marine Ecology-Progress Series* **176**, 165-
787 178.
788 Moens T, Vincx M (1997) Observations on the feeding ecology of estuarine nematodes. *Journal of the*
789 *Marine Biological Association of the United Kingdom* **77**, 211-227.
790 Moens T, Vincx M (1998) On the cultivation of free-living marine and estuarine nematodes.
791 *Helgoland Marine Research* **52**, 115-139.
792 Murfin KE, Dillman AR, Foster JM*, et al.* (2012) Nematode-Bacterium Symbioses-Cooperation and
793 Conflict Revealed in the "Omics" Age. *Biological Bulletin* **223**, 85-102.
794 Nascimento FJA, Naslund J, Elmgren R (2012) Meiofauna enhances organic matter mineralization in
795 soft sediment ecosystems. *Limnology and Oceanography* **57**, 338-346.

796 Perez-Portela R, Almada V, Turon X (2013) Cryptic speciation and genetic structure of widely
797 distributed brittle stars (Ophiuroidea) in Europe. *Zoologica Scripta* **42**, 151-169.
798 Pfenninger M, Schwenk K (2007) Cryptic animal species are homogeneously distributed among taxa
799 and biogeographical regions. *BMC Evolutionary Biology* **7**, 121.
800 Ristau K, Steinfartz S, Traunspurger W (2013) First evidence of cryptic species diversity and significant
801 population structure in a widespread freshwater nematode morphospecies (Tobrilus gracilis).
802 *Molecular Ecology* **22**, 4562-4575.
803 Segata N, Izard J, Waldron L*, et al.* (2011) Metagenomic biomarker discovery and explanation.
804 *Genome Biology* **12**.
805 Seymour MK, Wright KA, Doncaster CC (1983) The action of the anterior feeding apparatus of
806 *Caenorhabditis elegans* (Nematoda, Rhabditida). *Journal of Zoology* **201**, 527-539.
807 Sison-Mangus MP, Jiang S, Tran KN, Kudela RM (2014) Host-specific adaptation governs the
808 interaction of the marine diatom, Pseudo-nitzschia and their microbiota. *ISME J* **8**, 63-76.
809 Steyaert M, Garner N, van Gansbeke D, Vincx M (1999) Nematode communities from the North Sea:
810 environmental controls on species diversity and vertical distribution within the sediment.
811 *Journal of the Marine Biological Association of the United Kingdom* **79**, 253-264.
812 Sudhaus W, Kiontke K (2007) Comparison of the cryptic nematode species *Caenorhabditis brenneri* sp
813 n. and *C. remanei* (Nematoda : Rhabditidae) with the stem species pattern of the
814 *Caenorhabditis elegans* group. *Zootaxa*, 45-62.
815 Svanback R, Bolnick DI (2007) Intraspecific competition drives increased resource use diversity within
816 a natural population. *Proceedings of the Royal Society B-Biological Sciences* **274**, 839-844.
817 Team RDC (2008) R: A language and environment for statistical computing. R Foundation for
818 Statistical Computing, Vienna, Austria.
819 Tietjen JH, Lee JJ (1975) Axenic culture and uptake of dissolved organic substances by the marine
820 nematode, *Rhabditis marina* Bastian. *Cahiers de Biologie Marine* **XVI**, 685 - 693.
821 Vanaverbeke J, Gheskiere T, Vincx M (2000) The meiobenthos of subtidal sandbanks on the Belgian
822 Continental Shelf (Southern Bight of the North Sea). *Estuarine Coastal and Shelf Science* **51**,
823 637-649.
824 Wieser W (1953) Die Beziehung zwischen Mundhöhlengestalt, Ernährungsweise und Vorkommen bei
825 freilebenden marinen Nematoden. *Arkiv för Zoologi* **4**, 439-484.
826 Zhang J, Kobert K, Flouri T, Stamatakis A (2014) PEAR: a fast and accurate Illumina Paired-End reAd
827 mergeR. *Bioinformatics* **30**, 614-620.
828 Zoetendal EG, Akkermans ADL, De Vos WM (1998) Temperature gradient gel electrophoresis analysis
829 of 16S rRNA from human fecal samples reveals stable and host-specific communities of
830 active bacteria. *Applied and Environmental Microbiology* **64**, 3854-3859.

831

VI. Data accessibility

The raw sequence data of the two runs on the 454 GS FLX system are available in the Short Read Archive at NCBI under accession number SRP064694. The raw sequences of the MiSeq run are available under accession number SRP064727.

VII. Author contributions

SD collected and analyzed the 454 data and wrote the manuscript, NDM performed the food experiment and analyzed the MiSeq data, AR performed the molecular analyses, WKT conceived the study and along with SC and HB outlined opportunities to investigate meiobenthic diets using amplicon sequencing, TM and NDM critically revised the manuscript, and all authors contributed to the final version of the manuscript.

VIII. Supporting information

Appendix S1: Field distribution of four cryptic *Litoditis marina* species (Pm1, Pm2, Pm3 and Pm4) in the Scheldt estuary in The Netherlands in four consecutive seasons. Figure adapted from Derycke et al. (2006).

Appendix S2: Primer sequences used to amplify the 16S rRNA gene of 18 *Litoditis marina* specimens in two runs on $1/8^{th}$ of a plate of the 454 GS FLX Titanium system. Adaptor, midtag and primer sequences for the forward and reverse datasets are given.

Appendix S3: Summary of the sequence data from the two 454 runs performed on 18 field specimens

Appendix S4: Summary of the analyses to investigate variability between the technical replicates.

Appendix S5: Figures related to alpha diversity and beta diversity measurements of the forward dataset generated using the 454 platform of the field specimens.

Appendix S6: Rank abundance plots of the forward and reverse datasets generated with the 454 platform of the field specimens.

Appendix S7: The core OTUs of the forward and reverse datasets that are present in 100% of the specimens of species Pm1, Pm2 and Pm3 from the field. OTUs identified with LeFSe are indicated in bold. For the reverse dataset, OTUs were generated using 97% and 99% similarity.

Appendix S8: Summary of the sequence data from the MiSeq run on the specimens of the food experiment. Summary of the sequence data from a separate MiSeq run containing three biological replicas of the *E. coli* suspension are also provided.

Appendix S9: Detailed description of the taxonomic composition at the phylum, class and family level for the specimens of the food experiment.

Appendix S10: Graphs of alpha diversity (rarefaction curves of number of OTUs and Shannon Index, rank abundance plots) of the specimens of the food experiment.

Appendix S11: List of biomarker taxa identified by LeFSe for Pm1 and Pm3 from the food experiment. OTU ID and taxonomic assignment using Greengenes are included.

Appendix S12: SEM pictures of nematode specimens from Pm1, Pm2 and Pm3 with bacteria attached to the cuticula.

Appendix S13: Number of shared OTUs between each nematode specimen and each replica of the bacterial mix.

874 Appendix S14; Table with OTU IDs from the forward (sheet 1) and reverse dataset (sheet 2)
875 for the field specimens, along with their frequency in each specimen, the taxonomic
876 assignment using Uclust or Blast, and the quality score of the taxonomic assignment.

877 Appendix 15: Fasta file with representative sequences of OTUs from the forward dataset from
878 the field specimens

879 Appendix 16: Fasta file with representative sequences of OTUs from the reverse dataset from
880 the field specimens

881 Appendix 17: Table with OTU IDs from the MiSeq dataset for the specimens of the food
882 experiment, along with their frequency in each specimen, the taxonomic assignment using
883 Uclust, and the quality score of the taxonomic assignment.

884 Appendix 18: Fasta file with representative sequences from OTUs from the MiSeq run for the
885 specimens of the food experiment.

886

IX Tables

Table 1: Summary of the Permdisp and Permanova statistics between the microbiomes of the nematode species Pm1, Pm2 and Pm3. Analyses were done on the Forward and Reverse datasets using all OTUs or only those OTUs with relative frequency in the rarefied dataset ≥ 1%. For the pairwise comparisons, significant p-values after Bonferroni correction are indicated in bold.

		All OTUs		OTUs > 1%	
Forward dataset		F	p value	F	p value
PERMDISP		0.51	0.61	0.75	0.49
Overall PERMANOVA		1.79	0.007	1.76	0.04
Pairwise test	Pm1-Pm2	1.55	0.03	1.34	0.19
Pairwise test	Pm1-Pm3	2.21	**0.008**	2.65	**0.016**
Pairwise test	Pm2-Pm3	1.61	0.062	1.37	0.23
Reverse dataset					
PERMDISP		1.73	0.211	1.69	0.22
Overall PERMANOVA		1.62	0.001	1.90	0.012
Pairwise test	Pm1-Pm2	1.40	0.032	1.46	0.11
Pairwise test	Pm1-Pm3	2.11	**0.002**	2.88	**0.004**
Pairwise test	Pm2-Pm3	1.36	0.074	1.50	0.13

Table 2: Summary of the Permdisp and Permanova statistics between the microbiomes of the four food experiment treatments (Pm1B, Pm1E, Pm3B and Pm3E) for the dataset containing all OTUs and for the core OTUs. For the pairwise comparisons, significant p-values after Bonferroni correction are indicated in bold.

		All OTUs		Core Genome	
Food experiment		Pseudo-F	p value	Pseudo-F	p value
PERMDISP	species	9.04	<0.001	7.11	0.011
	food	2.94	0.095	1.57	0.22
	species*food	6.80	<0.001	6.65	0.001
Overall PERMANOVA	species	10.97	0.001	16.56	0.001
	food	3.10	0.005	3.59	0.008
	species*food	2.02	0.049	2.46	0.043
Pairwise test	Pm1B vs Pm1E	1.65	0.236	1.62	0.13
Pairwise test	Pm3B vs Pm3E	3.98	**0.004**	5.50	**0.001**
Pairwise test	Pm1B vs Pm3B	8.78	**0.004**	14.71	**0.001**
Pairwise test	Pm1E vs Pm3E	4.81	**0.004**	6.1	**0.002**

X. Figures

Figure 1. Relative composition of Proteobacteria for each of the 18 nematode specimens. Reads are from the Reverse dataset. A/Class level; B/ Family level Gammaproteobacteria, the eight most abundant taxa are shown, the 14 remaining taxa are pooled in a "Low Frequency Group"; C/ Family level Alphaproteobacteria, the six most abundant taxa are shown, the nine remaining taxa are pooled in a "Low Frequency Group".

Figure 2: Relative taxonomic composition of bacteria for each nematode specimen at the family level. Reads are from the Reverse dataset. A/ Actinobacteria, the seven most abundant families are shown, the remaining ten families are pooled in a "Low Frequency Group". B/ Bacteriodetes.

Figure 3: Rarefaction curves of the number of observed OTUs at 97% sequence identity clustering (A) and Shannon index (B) for each species for the Reverse dataset. Error bars were calculated from the variance of the respective parameter drawn in 10 randomizations at each sample size.

Figure 4: Principal coordinates analysis plot based on Generalized Unifrac distances between 18 nematode specimens after rarefaction at 600 sequences per specimen of the reverse dataset from the 454 platform. Intraspecific distances for Pm1 (black), Pm2 (blue) and Pm3 (red) are encircled.

Figure 5: Number of reads assigned to the core OTUs of Pm1, Pm2 and Pm3 in each of the 18 specimens from the rarefied reverse dataset. Legend reflects OTU name followed by the name of the species in which they were the core (eg 4334053Pm1Pm2 indicates that OTU 4334053 was present in all six specimens of Pm1 and in all six specimens of Pm2).

Figure 6: Taxonomic assignment of MiSeq reads at the family level for A/ the food experiment and B/ three biological replicas of the *E. coli* suspension. For the food experiment, the 15 most abundant families are shown, the remaining families are pooled in a "Low Frequency Group". Pm1B1-10: 10 biological replicas of Pm1 fed the bacterial mixture; Pm1E1-10: 10 biological replicas of Pm1 fed *E. coli*; Pm1C1-2: two biological replicas of the agar from Pm1 stock cultures; Pm3B1-10: 10 biological replicas of Pm3 fed the bacterial mixture; Pm3E1-10: 10 biological replicas of Pm3 fed *E. coli*; Pm3C1-2: two biological replicas of the agar from Pm3 stock cultures; bactmixa-b: two biological replicas of the bacterial mix. Vertical grey lines denote the different food treatments, stock cultures and bacterial mix. EcoliA-EcoliC: three biological replica's of the *E. coli* suspension. The "Low Frequency Group" contains 16 families.

Figure 7: Principal coordinates analysis plot of the Generalized Unifrac distances for the two species (Pm1 black/grey and Pm3 red/pink) and the two food treatments. E = *E. coli* (grey/pink) and B = bacterial mixture (black/red).